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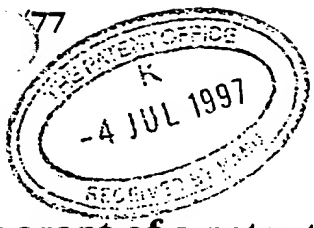

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OXFORD BIOMEDICA (UK) LIMITED
Medawar Centre
Robert Robinson Avenue
The Oxford Science Park
OXFORD
OX4 4GA

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — UNITED KINGDOM

7223522001

4. Title of the invention — THERAPEUTIC GENES

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Signature Gervis Hewlett & Perkins Agents for the Applicant Date 04-07-97

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THERAPEUTIC GENES

The perceived potential of monoclonal antibody-based therapies for treatment of neoplastic disease has not been fully realised (reviewed in Scheinberg and Chapman 1995, In Monoclonal antibodies (ed. Birch and Lennox) Chapter 2.1; George et al., 1994 Immunol. Today 15; 559-561). Consequently, monoclonal antibodies have been conjugated to radioisotopes, cytotoxic drugs or toxins in an attempt to improve efficacy. However, clinical trials with such conjugates have generally led to disappointing results. One of the principal reasons for the lack of efficacy of antibodies and antibody conjugates in the treatment of solid tumours is the poor penetration of solid tumours by immunoglobulins and other proteins such as immunotoxins of high molecular weight (eg. Juweid et al. 1992, Cancer Res. 52; 5144-5153; Epenetos et al. 1986 Cancer Res. 46; 3183-3191). Other reasons for lack of efficacy include the non-specific toxicity, immunogenicity and inappropriate pharmacokinetics of many immunotoxins and antibody-radionuclide conjugates introduced into the systemic circulation (reviewed in Scheinberg and Chapman 1995. In Monoclonal antibodies (ed. Birch and Lennox) Chapter 2.1).

In contrast to the general lack of in vivo efficacy, many monoclonal antibodies show pronounced ability to inhibit the growth of tumour cells in certain *in vitro* assays (reviewed in Sandlie and Michaelsen 1996 In Antibody engineering: a practical approach. Ed McCafferty et al. Chapter 9). It is well established that binding of specific antigen by an antibody can lead to activation of a variety of effector functions mediated via the Fc portion of the antibody heavy chain. The Fc regions of different immunoglobulin classes mediate different effector functions, including activation of complement cascades and binding to Fc receptors on various immune effector cells (Duncan et al. 1988 Nature 332; 563 and 738). In *in*

vitro assays, engagement of Fc receptors present on immune effector cell by antibody bound to tumour target cells can lead to destruction of the target cell by a variety of mechanisms collectively termed antibody dependent cellular cytotoxicity (ADCC). For example engagement of Fc-receptors for IgG, on human monocytes and macrophages, neutrophils and natural killer (NK) cells by antibodies of the IgG1 and IgG3 and to a much lesser extent IgG2 and IgG4 sub-classes, stimulates ADCC (Munn et al 1991 Cancer Res. 51; 1117-1123; Primus et al., 1993 Cancer Res. 53; 3355-3361). However, the relatively poor ability of such antibodies to destroy tumours *in vivo* suggests that ADCC does not play a significant role in many of the current antibody – based therapies (George et al., 1994 Immunol. Today 15; 559-561). There are several possible reasons for this, including the poor penetration of antibodies into solid tumours (Yuan et al. 1995 Cancer Res. 55; 3752-3756) and the fact that the majority of the high-affinity receptor (FcγRI) molecules present on macrophages are normally occupied by serum IgG which will be poorly competed by specific antibody (Munn et al 1991 Cancer Res. 51; 1117-1123).

It has previously been shown that tumour cells transduced with genes encoding monoclonal antibodies can participate in ADCC reactions mediated by xenogeneic NK cells *in vitro* (Primus et al. 1993 Cancer Res. 53: 3355-3361). However, NK cells play little role in the destruction of tumour cells *in vivo*, in part because their killing functions are inhibited by the presence of self MHC Class I on autologous tumour cells (Correa and Raulet 1995 Immunity 2; 61-71).

It has also been postulated that tumour infiltrating lymphocytes (TILs) could be used as a vehicle to deliver antibody genes to a tumour to secrete anti-tumour antibodies at the tumour site (Tsang et al 1993 J. Immunother. 13; 143-152.) However, *ex vivo* transduction of TILs followed by autologous transplantation using marker genes has shown that isolated TILs show no specific homing mechanism which could allow them

to return to tumour deposits (Economou et al 1996 J. Clin. Invest. 97; 515-521) and so any such approach is of limited value. Transduction of a gene encoding a single-chain immunotoxin into human lymphokine – activated T-cells (LAK cells) has also been reported (Chen et al 1997 Nature 385, 78-80). In addition to the problems of re-introducing the LAK cells to the site of the tumour, such an approach also suffers from the potential drawbacks associated with being restricted to *ex vivo* use. These include the necessity of culturing the T-cells in high levels of a cytokine such as IL-2 to generate LAK cells with consequent problems in generating sufficient cells for therapy.

The present invention relates to the use of genetic vectors to deliver therapeutic genes encoding secreted tumour binding proteins (TBPs) to the interior of a tumour mass and identifies ways to target expression of TBPs to the interior of the tumour. Expression of the gene or genes encoding the TBP within the tumour mass then leads to local production of TBP with consequent reduction of tumor growth, survival or dissemination by a variety of mechanisms. Because the TBP is secreted, TBP produced by transduced cells can act not only on the transduced cell but on neighbouring tumour cells as well and hence achieve a bystander effect.

There are a number of cell types present within a tumor mass in addition to the cancerous cells. These can include cells of the tumour vasculature (eg endothelial cells) and immune cells which infiltrate the tumour, such as tumour-infiltrating lymphocytes (TIL) and macrophages (Normann 1985 Cancer Metastasis Re. 4:277-291; Leek et al 1996 Cancer Res. 56: 4625-4629). Any of these cell types can be targeted for expression of the TBP and can serve as a local factory within the tumour for production of TBP. Preferably, the cells in the tumour mass which are used to produce the TBP are the cancerous cells, endothelial cells or macrophages.

The TBP comprises one or more binding domains capable of binding to one or more tumour-associated cell-surface molecules (TACSMs) which are present on the cancerous cells. Thus the TBP, produced from one or more of the cell types within the tumour mass is secreted and is directed to the cancerous cells by its affinity for the TACSM. The TACSM is selectively present on a restricted number of cell types. Thus the amount of TACSM present on the majority of the cancerous cells within the tumour mass is higher than on surrounding tissues. Preferably, the TACSM is detectably present only on tumour cells and a limited number of other tissue types in the individual containing the tumour. More preferably, the TACSM is essentially tumour-specific in the individual containing the tumour.

The one or more binding domains of the TBP may consist of, for example, a natural ligand for a TACSM, which natural ligand may be an adhesion molecule or a growth-factor receptor ligand (eg epidermal growth factor), or a fragment of a natural ligand which retains binding affinity for the TACSM. Alternatively, the binding domains may be derived from heavy and light chain sequences from an immunoglobulin (Ig) variable region. Such a variable region may be derived from a natural human antibody or an antibody from another species such as a rodent antibody. Alternatively the variable region may be derived from an engineered antibody such as a humanised antibody or from a phage display library from an immunised or a non-immunised animal or a mutagenised phage–display library. As a second alternative, the variable region may be derived from a single-chain variable fragment (scFv). The TBP may contain other sequences to achieve multimerisation or to act as spacers between the binding domains or which result from the insertion of restriction sites in the genes encoding the TBP, including Ig hinge sequences or novel spacers and engineered linker sequences.

The TBP may comprise, in addition to one or more immunoglobulin variable regions, all or part of an Ig heavy chain constant region and so may comprise a natural whole Ig, an engineered Ig, an engineered Ig-like molecule, a single-chain Ig or a single-chain Ig–like molecule. Alternatively, or in addition, the TBP may contain one or more domains from another protein such as a toxin.

In one aspect of the invention, there is provided a gene delivery system for targeting one or more genes encoding a TBP to a tumour, comprising a genetic vector encoding a TBP and an *in vivo* gene-delivery system. The gene delivery system may be a non-viral gene delivery system such as DNA compacted with a DNA-compaction agent, or a liposome or immunoliposome which may contain DNA compacted with a DNA–compaction agent (such as a poly-lysine). The vector may be a plasmid DNA vector. Alternatively the vector may be a recombinant viral vector such as an adenovirus vector, an adeno–associated virus (AAV) vector, a herpes-virus vector or a retroviral vector in which case gene delivery is mediated by viral infection of a target cell. Preferably the vector is a recombinant retroviral vector, which may be a targeted retroviral vector. Preferably, the retroviral vector is resistant to human complement, for example by production in a human cell line. In any event, the vector will contain a promoter to direct expression of the or each therapeutic gene and may contain additional genetic elements for the efficient or regulated expression of TBP genes, including enhancers, translation initiation signals, internal ribosome entry sites (IRES), splicing and polyadenylation signals. The promoter and/or enhancer may be tissue-restricted in its activity. For example a tumour-specific promoter-enhancer, such as a 5T4 antigen gene promoter-enhancer or the CEA-gene promoter-enhancer may be used. Alternatively, or additionally, an element or elements for regulated expression may be present, such as a hypoxia regulated enhancer. An example of a hypoxia regulated expression element (HRE)

is a binding element for the transcription factor HIF1. The enhancer elements or elements conferring regulated expression may be present in multiple copies. Preferably, expression of the or a therapeutic gene is inducible by hypoxia (or low oxygen supply) such as may be found in a tumour mass. Most preferably, the promoter and/or enhancer directing expression of the therapeutic gene contains both hypoxia–responsive elements and elements which give higher expression in tumour cells than in neighbouring non-tumour cells.

Additional vector components will be provided for other aspects of vector function such as vector maintenance, nuclear localisation, replication, and integration as appropriate using components which are well known in the art.

In a preferred embodiment of this aspect of the invention, a retroviral vector is provided for *in vivo* delivery of the gene or genes encoding the TBP to the tumour. Suitable retroviral vectors are known in the art (see for example Gunzberg and Salmons 1996 In Gene Therapy ed. Lemoine and Cooper. Bios; and Cosset et al. 1995 J. Virol. 69; 7430-7436). In a particularly preferred embodiment, expression of the TBP may be enhanced in the hypoxic regions of the tumour by the inclusion of hypoxia regulated genetic elements in the retroviral vector. In this case, the hypoxia–regulated elements may be inserted into one or both of the retroviral LTRs in place of the LTR enhancer or in another position in the vector, by standard molecular biology techniques. The gene or genes encoding the TBP may be expressed from a promoter-enhancer which leads to enhanced expression in the tumour cells compared with neighbouring non-tumour cells or is preferably essentially tumour–specific. Examples of suitable promoters include the promoter-enhancer of the gene for 5T4 antigen, the promoter–enhancer of the MUC1 gene or the CEA gene.

In a second aspect of the invention there is provided a method of treating cancer comprising administering the TBP gene or genes in a gene delivery system of the first aspect of the invention either systemically or directly to the site of a tumour.

In a third aspect of the invention, is provided a gene delivery system for introducing one or more genes encoding a TBP into cells of the myeloid haematopoietic cell lineage either *in vivo* or *ex vivo*. Preferably the myeloid cells are of the monocyte–macrophage lineage or a precursor of such cells such as a CD34-positive stem cell. For *ex vivo* delivery, the genes can be inserted into a plasmid vector and delivered by one of a variety of DNA transfection methods including electroporation, DNA biolistics, lipid–mediated transfection or compacted DNA–mediated transfection. Alternatively a viral vector can be used to transduce myeloid cells or CD34-positive stem cells *ex vivo*, such as an adenovirus vector, a retroviral vector or a lentiviral vector. The vector will contain a promoter to direct expression of the or each therapeutic gene and may contain additional genetic elements for efficient or regulated expression including enhancers, translation initiation signals, internal ribosome entry sites (IRES), splicing and polyadenylation signals. The promoter, or an enhancer or splicing signals may be tissue-restricted and preferentially active in mononuclear phagocytes such as macrophages. The promoter and/or enhancer may contain elements for regulated expression such as a hypoxia-regulated enhancer. An example of a hypoxia regulated expression element is HIF1 transcription factor response element. Such an element may be present in multiple copies. Examples of hypoxia–regulated promoters and enhancers include those from the enolase gene, the erythropoietin gene, and genes encoding glycolytic enzymes (Semenza et al., 1994 J. Biol. Chem 269; 23757-23763) such as the PGK gene. Isolated HREs can be multimerised in order to increase the response to hypoxia. Additional vector components may be provided for other aspects

of vector function such as vector maintenance, nuclear localisation, replication and integration as appropriate using components which are well known in the art.

After introduction of the vector into the cells ex vivo, the cells can be re-introduced into the patient directly or they can be stimulated to differentiate along the monocyte-macrophage differentiation pathway using appropriate combinations of cytokines and growth factors prior to re-introduction into the patient. CD34-positive cells are stimulated to differentiate using cytokines including IL-3, GMCSF and MCSF. Monocytes are differentiated either by culture attached to plastic or using GMCSF either alone or in combination with other cytokines including MCSF.

For introduction of therapeutic genes into myeloid cells or CD34-positive stem cells *in vivo*, a suitable *in vivo* delivery system can be used to deliver the transcription units described above. The gene delivery system may be a non-viral gene delivery system such as DNA compacted with a DNA-compaction agent, or a liposome or immunoliposome which may contain DNA compacted with a DNA–compaction agent. Alternatively the vector may be a recombinant viral vector such as a targeted adenovirus vector, an adeno–associated viral (AAV) vector, a herpes-virus vector or a retroviral vector such as a lentiviral vector. Preferably the vector is a targeted recombinant retroviral vector, which is preferably resistant to human complement, for example by preparation of the vector from a human packaging cell line.

CD34-positive stem cells can also differentiate to form endothelial cells (Ashara et al. 1997 Science 275; 964-967). Such a route of differentiation for CD34 positive stem cells containing TBP encoding genes according to the invention is envisaged in addition to differentiation to form monocytes and macrophages.

Additional vector components may be provided for other aspects of vector function such as vector maintenance, nuclear localisation, replication, and integration as appropriate using components which are well known in the art.

In a preferred embodiment of this aspect of the invention, a plasmid vector or a retroviral vector carrying a gene encoding a TBP under the control of a hypoxia regulated promoter or a promoter preferentially active in macrophages is introduced into autologous peripheral blood monocytes. The transfected monocytes are re-introduced into the patient where they migrate to the hypoxic regions of tumours permitting enhanced production of the TBP in the interior of the tumour mass. The macrophages are optionally treated with cytokines prior to re-injection into the patient. Alternatively or additionally the vector may include DNA sequences capable of expressing a cytokine gene such as a gene for IFNγ, CSF-1 or GM-CSF in order to elicit the differentiation of the transfected cells. The cytokine gene may also be regulated by genetic elements which show enhanced activity at the site of the tumour.

In a fourth aspect of the invention, there is provided a method for treating cancer in a human or non-human mammal, comprising withdrawing an amount of blood from an individual suffering from cancer, preparing from the blood a cell preparation enriched in monocytes and macrophages or their stem-cell progenitors, introducing TBP genes into the cell preparation using a gene delivery system of the third aspect of the invention so as to bring about transfection or transduction of the monocytes and macrophages, or their stem-cell progenitors with the TBP genes, and re-introducing the transfected or transduced cells either systemically or directly to the site of the tumour. The cell preparations may optionally be treated with cytokines prior to reintroduction in order to elicit differentiation towards active macrophages.

In a fifth aspect of the invention is provided a method for treating cancer in a mammal, comprising administering to an individual a gene delivery system of the third aspect of the invention capable of expressing a TBP in cells derived from a myeloid origin.

In a further aspect of the invention there is provided a genetic vector comprising a therapeutic gene or genes encoding a TBP, operably linked to an expression regulatory element selectively functional in a cell type present within a tumour mass. The TBP in this aspect of the invention inhibits tumour function by binding to a TACSM having an essential role in tumour cell survival or dissemination. The TACSM in this aspect of the invention may be a cell surface molecule which has a role in tumour cell growth, migration or metastasis, and is present on cancerous cells or on another cell type within the tumour mass. Preferably the TACSM is present on cancerous cells or tumour vasculature or on macrophages and is a molecule such as a growth-factor receptor, a plasminogen activator, a metalloproteinase or the 5T4 antigen. The gene or genes encoding the TBP may be delivered to the interior of the tumour by any of the routes described in the first two aspects of the invention. Binding of the TBP to the corresponding TACSM blocks the function of the TACSM and thereby leads to inhibition of growth, migration or metastasis of the tumour.

In a yet further aspect of the invention, a genetic vector comprising a therapeutic gene or genes is delivered to the interior of the tumour wherein the therapeutic gene encodes a TBP, which additionally contains one or more effector domains. The effector domain or domains may be activated on binding of the TBP to a TACSM leading to inhibition of tumour cell proliferation, survival or dissemination. The TACSM in this aspect of the invention is a cell surface molecule for which a specific TBP is available such as a tumour specific carbohydrate moiety, an oncofoetal antigen, a mucin, a growth-factor receptor or another glycoprotein. The TACSM is preferably an antigen restricted in its tissue distribution and

found predominantly on the tumour cells and on the majority of cells within the tumour. Alternatively, the TACSM is present on tumour macrophages or the tumour vasculature. The TACSM is most preferably not shed from the cell surface to an appreciable extent.

The effector domain may possess enzymatic activity and may be for example a pro-drug activating enzyme. Examples of TBPs containing effector domains with enzyme activity include antibody - enzyme conjugates or fusions. Antibody - enzyme conjugates have been described including conjugates with alkaline phosphatase (Senter *et al.*, 1988 Proc. Natl. Acad. Sci. 85: 4842-4846); carboxypeptidase G2 (Bagshawe *et al.* 1988 Br. J. Cancer 58: 700-703); β-lactamase (Shepherd *et al* 1991 Bioorg. Med. Chem. Lett. 1:21-26); and Penicillin -V- amidase (Kerr *et al.* 1990 Cancer Immunol. Immunother. 31: 202-206). Antibody - enzyme fusions have also been described (Goshorn *et al* 1993 Cancer Res 53: 2123-2127; Wels *et al* 1992 Bio/Technology 10: 1128-1132). Each of these examples can be used in this aspect of the invention. Additional or alternative enzymes which may be included in TBP-enzyme fusions include human Carboxypeptidase A1 or a mutant thereof (Smith *et al* 1997 J. Biol. Chem. 272: 15804-15816); cytosine deaminase (Mullen *et al.* 1994 Cancer Res. 54: 1503-1506); HSV thymidine kinase (Borrelli *et al.* 1988 Proc . Natl. Acad. Sci. 85: 7572-7576.); nitroreductase; P450-Reductase and a P450.

Preferably the pro-drug activating enzyme domain or domains are genetically fused to the C-terminus of an immunoglobulin or immunoglobulin domain such as a scFv or a single-chain antibody or Fab-fragment. In a particularly preferred embodiment of this aspect of the invention, the immunoglobulin domain or domains are human or humanised and the enzyme is a human enzyme such as a Carboxypeptidase a P450 or P450-Reductase. The enzyme may be a

mutant enzyme which converts a pro-drug more efficiently than does the native human enzyme.

In each case, a suitable pro-drug is used in the treatment of the patient in combination with the appropriate pro-drug activating enzyme. Examples of pro-drugs include etoposide phosphate (used with alkaline phosphatase Senter *et al.*, 1988 Proc. Natl. Acad. Sci. 85: 4842-4846); 5-fluorocytosine (with Cytosine deaminase Mullen *et al.* 1994 Cancer Res. 54: 1503-1506); Doxorubicin-N-p-hydroxyphenoxyacetamide (with Penicillin-V-Amidase (Kerr *et al.*1990 Cancer Immunol. Immunother. 31: 202-206); Para-N-bis(2-chloroethyl) aminobenzoyl glutamate (with Carboxypeptidase G2); Cephalosporin nitrogen mustard carbamates (with β-lactamase); SR4233 (with P450 Reducase); Ganciclovir (with HSV thymidine kinase, Borrelli *et al.* 1988 Proc . Natl. Acad. Sci. 85: 7572-7576) mustard pro-drugs with nitroreductase (Friedlos *et al.* 1997J Med Chem 40: 1270-1275) and Cyclophosphamide (with P450 Chen *et al.* 1996 Cancer Res 56: 1331-1340).

Alternatively, the effector domain may be a non-enzyme domain. Examples of non-enzyme effector domains include toxins such an exotoxin from a pseudomonad bacterium, all or part of a cytokine such as IL-2 or IFNγ, or effector domains from immunoglobulin heavy chains.

In a preferred embodiment of this aspect of the invention, the TBP contains an effector domain capable of activating macrophage FcγR I, II or III receptors. On binding of the TBP to antigen on the tumour cells, macrophages present within the hypoxic regions of the tumour are activated to destroy the tumour cells directly by phagocytosis or ADCC or are activated to secrete pro-inflammatory cytokines which serve to enhance the natural immunological response to the tumour. The TBP may contain an Fc region from an immunoglobulin, a mutant Fc region, a receptor-binding fragment of the Fc region or may contain another FcR-binding domain. Preferably the TBP contains an effector domain

which additionally confers protein stability *in vivo*, such as an intact Fc region from an IgG, more preferably from human IgG1, or IgG3 and most preferably from human IgG1.

In a particularly preferred embodiment of this aspect of the invention, the TBP is a Sab containing a human IgG1 constant region and a binding domain which recognises the 5T4 antigen.

The effector domain may be encoded by a portion of a cDNA fused in-frame to the DNA encoding the tumour-binding domain. Alternatively a genomic fragment containing introns may be used such as a human IgG1 heavy chain constant region genomic fragment.

Introduction of TBP-encoding genes into monocytes or macrophages may be combined with further treatments to elicit macrophage differentiation and activation. For example, cells maintained ex vivo may be treated with cytokines such as IFNγ, CSF-1 or GM-CSF prior to re-introduction into the patient. Alternatively, genes encoding these cytokines may be introduced into the monocytes/macrophages in the same or a different vector from the TBP genes *in vivo* or *ex vivo*. Consequently in a still further aspect of the invention there is provided a method of treating cancer in a mammal which comprises administering to an individual a combination of a cytokine or a cytokine–encoding gene and one or more TBP genes according to any of the previous aspects of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 – shows a DNA sequence encoding a 5T4 scFv, designated 5T4scFv.1. The sequence of the mature secreted protein is given.

Figure 2 – shows the cDNA sequence encoding 5T4Sab1. The sequence begins with a HindIII restriction site followed by a translation initiation signal and a signal peptide.

DETAILED DESCRIPTION

In accordance with the invention, standard molecular biology techniques may be used which are within the level of skill in the art. Such techniques are fully described in the literature. See for example; Sambrook et al. (1989) Molecular Cloning; a laboratory manual; Hames and Glover (1985 – 1997) DNA Cloning: a practical approach, Volumes I-IV (second edition). Methods for the engineering of immunoglobulin genes in particular are given in McCafferty et al (1996) Antibody engineering: a practical approach.

A genetic "vector" is a genetic element (such as a plasmid, chromosome, artificial chromosome or a virus) which functions to transfer a segment of heterologous DNA into a target cell. Once within the target cell, the vector may serve to maintain the heterologous DNA within the cell or may act as a unit of DNA replication.

A "promoter" is a DNA sequence capable of binding an RNA polymerase enzyme within a cell and directing the initiation of transcription of a coding sequence at a specific site on the DNA.

An "enhancer" is a DNA sequence which binds to other protein components of the transcription initiation complex and thus facilitates the initiation of transcription directed by its associated promoter.

An "intron" is an intervening sequence of DNA within a gene which is removed by RNA splicing and so is not present in the mature messenger RNA and does not code for protein. Introns can be conditional or alternatively spliced in different cell types.

A "retrovirus" is a virus which contains genomic RNA which on entry into a host cell is converted to a DNA molecule by a reverse transcriptase enzyme. The DNA copy serves as a template for the production of new RNA genomes and virally encoded proteins necessary for the assembly of infectious viral particles. The term "recombinant retroviral vector" describes a DNA molecule which contains sufficient retroviral sequences to allow an RNA transcript of the vector to be packaged in the presence of essential retroviral proteins into a retroviral particle capable of infecting a target cell. Infection of the target cell includes reverse transcription and integration into the target cell genome. The term "recombinant retroviral vector" also covers a retroviral particle containing an RNA genome encoded by the DNA molecule. The retroviral vector will also contain non-viral genes which are to be delivered by the vector to the target cell. A recombinant retroviral vector is incapable of

independent replication to produce infectious retroviral particles. Usually, recombinant retroviral vector lacks functional *gag-pol* and/or *env* genes, or other genes encoding proteins essential for replication. A "targeted retroviral vector" is a recombinant retroviral vector whose ability to infect a cell or to be expressed in the target cell is restricted to certain cell types within the host organism. An example of targeted retroviral vectors is one with a genetically modified envelope protein which binds to cell surface molecules found only on a limited number of cell types in the host organism. Another example of a targeted retroviral vector is one which contains promoter and/or enhancer elements which permit expression of one or more retroviral transcripts in only a proportion of the cell types of the host organism.

An "envelope" protein is a viral protein which coats the viral particle and plays an essential role in permitting viral entry into a target cell.

"Transduction" is the process of using a viral vector to deliver a non-viral gene to a target cell. "Transfection" is a process using a non-viral vector to deliver a gene to a target mammalian cell.

"Immunoglobulin" is the term given to a member of a family of related multimeric proteins which are normally secreted from cells of the B-lymphocyte lineage of a vertebrate, whose function is to bind specifically with a region of a macromolecule identified as non-self. Immunoglobulins represent a major component of the immune response repertoire of the organism and are synonymous with "antibodies".

In its primary aspect the invention relates to the delivery of TBP–encoding genes to the site of a tumour. This has considerable advantages for therapeutic applications in which TBPs are indicated since it circumvents a number of problems associated with delivery of proteins systemically in man. Proteins are complex molecules which, of necessity, are produced from biological sources, most usually from genetically engineered organisms or cell cultures. The procedures for production of

TBPs are consequently complicated, labour intensive and costly. Furthermore, pharmacological properties and other aspects of the function of TBPs such as immunoglobulins derived from non-human biological sources may frequently differ in important ways from the activity of the corresponding natural human immunoglobulins produced in human cells. One major cause of such differences in activity is variations in the pattern of glycosylation of proteins derived from different species (reviewed in Bebbington 1995; In Monoclonal Antibodies: the second generation ed . H. Zola pg 165-181). Systemic administration of TBPs containing toxin domains can identify additional pharmacokinetic and toxicological problems (reviewed in Scheinberg and Chapman 1995. In Monoclonal antibodies (ed. Birch and Lennox) Chapter 2.1).

In contrast to the problems associated with production and delivery of proteins, the methods of the invention allow the delivery of genes to the site of the tumour, thus circumventing a number of production problems. The TBPs are thereby produced *in situ* in the autologous human cells, which serve as a local factory for the production of the gene-based therapeutic. This has significant advantages in minimising systemic toxicity. The activity of the protein is maximal since the glycosylation of the protein shows a human pattern appropriate to the individual being treated.

The methods of the invention can be used in conjunction with direct injection into the site of the tumour or systemic delivery of, for example targeted vectors or engineered myeloid cells or their progenitors. Systemic delivery may be particularly advantageous in a number of indications, particularly in the treatment of disseminated disease. In these cases the gene delivery system or engineered cells can be administered intravenously by bolus injection or by infusion in a suitable formulation. A pharmaceutically acceptable formulation may include an isotonic saline solution, a buffered saline solution or a tissue-culture medium. Additional

formulatory agents may be included such as preservative or stabilising agents.

The invention will now be further described by way of examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in any way to limit the scope of the invention.

EXAMPLES

Example 1 – Construction of 5T4 Sab and retroviral - vector delivery to tumour.

The trophoblast cell surface antigen, originally defined by monoclonal antibody 5T4 (Hole and Stern 1988 Br. J. Cancer 57; 239-246), is expressed at high levels on the cells of a wide variety of human carcinomas (Myers et al. 1994 J. Biol. Chem. 269; 9319-9324) but, in normal tissues of non-pregnant individuals, is essentially restricted to low level expression on a few specialised epithelia (Myers et al. *ibid.* and references therein). The 5T4 antigen has been implicated in contributing to the development of metastatic potential and therefore antibodies specifically recognising this molecule may have clinical relevance in the treatment of tumours expressing the antigen.

The cDNA encoding the murine 5T4 monoclonal antibody is cloned and sequenced by standard techniques (Antibody engineering: a practical approach ed McCafferty et al. 1996 OUP). The sequence of the

variable region of the antibody can be used to construct a variety of immunoglobulin-like molecules including scFvs. The coding sequence of a 5T4 scFv, 5T4scFv.1, is shown in Figure 1. In this molecule, the DNA sequence encodes the Vh from the mouse 5T4 monoclonal antibody followed by a 15 amino acid flexible linker and the Vl region of the mouse 5T4 antibody. The flexible linker encodes 3 copies of the amino-acid sequence gly-gly-gly-gly-ser and the DNA sequence similarity between the repeats has been minimised to avoid the risk of recombination between the repeats when plasmids containing them are grown in E. coli.

The DNA sequences shown in Figure 1 can also be used to construct a variety of single-chain antibodies (Sabs) by coupling scFv–encoding sequences to a sequence encoding a Fc region to form an in-frame fusion. A Sab is constructed using a series of DNA cassettes which can be independently varied to suit particular purposes.

Cassette 1 – Translation initiation signal and signal peptide

In order to achieve correct translation initiation and secretion from mammalian cells, the following sequence is used:

aagcttCCACCATGG G ATGGAGCTGT ATCATCCTCTTCTTGGTAGC AACAGCTACA GGTGTCCACT CC

This contains a convenient HindIII restriction site for cloning into expression vectors (lower case), the consensus translation initiation signal for mammalian cells (ANNATGPu) and the coding sequence for a signal peptide sequence from an immunoglobulin gene.

Cassette 2 – scFv

The sequence of the secreted portion of the 5T4scFv.1 is shown in Figure 1. This molecule can be represented as Vh – $(gly_4\text{-}ser)_3$ linker - Vl.

5T4 scFv2 consists of the 5T4 variable region sequences connected in the order Vl – flexible linker Vh. In this case the linker encodes the 20 amino-acid peptide $(gly_4\text{-}ser)_4$. A longer linker improves assembly of the scFv when the V-region segments are in this order. (Pluckthun et al in Antibody Engineering: a practical approach, ed McCafferty et al. 1996 OUP).

Cassette 3 – Heavy chain Constant region

The sequence of a human γ1 constant region genomic clone is given in Ellison et al. 1982 Nucl. Acids res. 10: 4071-4079. This sequence contains constant–region introns in addition to the coding sequence. This is fused in-frame to the 3'-end of one of the scFv sequences from Cassette 2. Vectors for convenient assembly of such constructs are described (Walls et al. 1993 Nucl. Acids Res. 21:2921-2929.

A cDNA of a 5T4 Sab, designated 5T4Sab1 is shown in Figure 2, containing cassettes 1, 2 and 3.

The variable region of the 5T4 monoclonal antibody can also be humanised by a number of techniques, which are known in the art, including grafting of the CDR region sequences on to a human backbone. These can then be used to construct an intact humanised antibody or a humanised Sab (see Antibody Engineering: a practical approach, ed McCafferty et al. 1996 OUP).

For expression of a 5T4-specific scFv or Sab in human cells, the coding sequence is inserted into the vector pCIneo (Promega) under the control of a strong promoter and polyadenylation signal. The translation initiation signal and immunoglobulin leader (signal peptide)

sequence from Cassette 1 at the 5'end of the coding region ensure efficient secretion of the scFv or Sab from mammalian cells.

For expression of an intact Ig, two separate translation cassettes are constructed, one for the heavy chain and one for the light chain. These are separated by an internal ribosome - entry site (IRES) from the picornavirus FMDV (Ramesh et al. 1996 Nucl. Acids Res. 24: 2697-2700. Alternatively, each cDNA is expressed from a separate copy of the hCMV promoter (Ward and Bebbington 1995 In Monoclonal Antibodies ed Birch and Lennox.Wiley-Liss).

For production of retrovirus capable of expressing 5T4 antibody or immunoglobulin-like molecules with 5T4 specificity, the gene encoding a 5T4-based Sab, or a dicistronic message encoding heavy and light chains, is inserted into a retroviral vector in which retroviral genomic transcripts are produced from a strong promoter such as the hCMV-MIE promoter. A suitable plasmid is pHIT111 (Soneoka et al. 1995 Nucl. Acids Res.23; 628-633) and the required gene is inserted in place of the LacZ gene using standard techniques. The resulting plasmid, pHIT-5T4.1 is then transfected into the FLYRD18 or FLYA13 packaging cell lines (Cosset et al. 1995 J. Virol. 69; 7430-7436) and transfectants selected for resistance to G418 at 1 mg/ml. G418-resistant packaging cells produce high titres of recombinant retrovirus capable of infecting human cells. The virus preparation is then used to infect human cancer cells and can be injected into tumours *in vivo*. The 5T4 Sab is then expressed and secreted from the tumour cells.

In pHIT111, the MoMLV LTR promoter-enhancer is used for expression of the therapeutic gene in the target cell. The vector can also be modified so that the therapeutic gene is transcribed from an internal promoter-enhancer such as one which is active predominantly in the tumour cells or one which contains a hypoxia regulated element. A

suitable promoter is a truncated HSV TK promoter with 3 copies of the mouse PGK HRE (Firth et al. 1994 Proc. Natl. Acad. Sci. 91: 6496-6500).

Example 2 – Transfection of macrophages / monocytes with an expression vector encoding TBP.

Peripheral blood mononuclear cells are isolated from human peripheral blood at laboratory scale by standard techniques procedures (Sandlie and Michaelsen 1996 In Antibody engineering: a practical approach. Ed McCafferty et al. Chapter 9) and at large scale by elutriation (eg Ceprate from CellPro). Adherent cells (essentially monocytes) are enriched by adherence to plastic overnight and cells can be allowed to differentiate along the macrophage differentiation pathway by culturing adherent cells for 1-3 weeks.

Monocytes and macrophages are transfected with an expression vector capable of expressing TBP in human cells. For constitutive high level expression, the TBP is expressed in a vector which utilises the hCMV-MIE promoter-enhancer, pCI (Promega). For hypoxia-induced expression, the hCMV promoter is replaced by a promoter containing at least one HRE. A suitable promoter is a truncated HSV TK promoter with 3 copies of the mouse PGK HRE (Firth et al. 1994 Proc. Natl. Acad. Sci. 91: 6496-6500).

A variety of transfection methods can be used to introduce vectors into monocytes and macrophages, including particle-mediated DNA delivery (biolistics), electroporation, cationic agent-mediated transfection (eg using Superfect, Qiagen). Each of these methods is carried out according to the manufacturer's instructions, taking into account the parameters to be varied to achieve optimal results as specified by the individual manufacturer. Alternatively, viral vectors may be used such as defective Adenovirus vectors (Microbix Inc or Quantum Biotechnologies Inc).

Example 3 – Assay for ADCC mediated by macrophages

Cells from primary human tumours or tumour cell lines which have been transduced with retrovirus expressing TBP are mixed with autologous or heterologous human macrophages, prepared as described in Example 2, for analysis of ADCC activity mediated by the TBP. Alternatively, macrophages engineered to produce TBP as described in Example 2 can be used to direct ADCC on non-transduced tumour cells.

The assay is carried out according to standard procedures (Sandlie and Michaelsen 1996 In Antibody engineering: a practical approach. Ed McCafferty et al. Chapter 9) with appropriate modifications. Briefly, the effector cells (macrophages or freshly isolated monocytes) are suspended at 3 x 10^6 cells / ml in the appropriate tissue culture medium (DMEM/Hepes, obtained from Life Technologies, containing 1% Foetal Calf Serum). 3 x 10^5 tumour target cells, labelled with ^{51}Cr are placed in each well of a round-bottomed microtitre plate in 0.1 ml culture medium. (Note the culture medium can include spent medium from the cells producing the TBP). 50 µl effector cells are added to the wells, the plate is centrifuged at 300g for 2 min and incubated at 37°C for varying periods (eg 4 h) in a tissue culture incubator. The supernatant is then harvested by centrifugation and counted in a gamma counter. Results are expressed as percent lysis relative to total chromium release from an equivalent sample of target cells lysed with 0.1% Tween-20. The effector: target cell ratio can be varied in the assay to produce a titration curve.

For the prior stimulation of macrophage differentiation or priming, cytokines are added to the cultures. IFNγ (Sigma) is added at between 100 and 5000 U/ml. CSF-1 or GM-CSF (Santa Cruz Biotechnology) can also be added at appropriate concentrations.

Example 4 – Analysis of efficacy in animal models

Human tumour–derived cell lines and tissues are cultured *in vivo* in genetically immunodeficient, "nude" mice according to well established techniques (see for example Strobel et al. 1997 Cancer Res. 57: 1228-1232; McLeod et al. 1997 Pancreas 14: 237-248). Syngeneic mouse models, in which a syngeneic tumour line is introduced into an immunocompetent mouse strain may also be used. These serve as suitable animal models for evaluating gene delivery systems of the invention. Vectors or engineered cells are administered systemically or directly into the tumour and tumour growth is monitored in treated and untreated animals. This system is used to define the effective dose range of the treatments of the invention and the most appropriate route of administration.

Figure 1

```
1   GAGGTCCAGC TTCAGCAGTC TGGACCTGAC CTGGTGAAGC CTGGGGCTTC
      E  V  Q    L  Q  Q    S  G  P  D    L  V  K    P  G  A

51  AGTGAAGATA TCCTGCAAGG CTTCTGGTTA CTCATTCACT GGCTACTACA
      S  V  K  I    S  C  K    A  S  G    Y  S  F  T    G  Y  Y

101 TGCACTGGGT GAAGCAGAGC CATGGAAAGA GCCTTGAGTG GATTGGACGT
      M  H  W    V  K  Q  S    H  G  K    S  L  E    W  I  G  R

151 ATTAATCCTA ACAATGGTGT TACTCTCTAC AACCAGAAAT TCAAGGACAA
      I  N  P    N  N  G    V  T  L  Y    N  Q  K    F  K  D

201 GGCCATATTA ACTGTAGACA AGTCATCCAC CACAGCCTAC ATGGAGCTCC
      K  A  I  L    T  V  D    K  S  S    T  T  A  Y    M  E  L

251 GCAGCCTGAC ATCTGAGGAC TCTGCGGTCT ATTACTGTGC AAGATCTACT
      R  S  L    T  S  E  D    S  A  V    Y  Y  C    A  R  S  T

301 ATGATTACGA ACTATGTTAT GGACTACTGG GGTCAAGTAA CCTCAGTCAC
      M  I  T    N  Y  V    M  D  Y  W    G  Q  V    T  S  V

351 CGTCTCCTCA GGTGGTGGTG GGAGCGGTGG TGGCGGCACT GGCGGCGGCG
      T  V  S  S    G  G  G    G  S  G    G  G  G  T    G  G  G

401 GATCTAGTAT TGTGATGACC CAGACTCCCA CATTCCTGCT TGTTTCAGCA
      G  S  S    I  V  M  T    Q  T  P    T  F  L    L  V  S  A

451 GGAGACAGGG TTACCATAAC CTGCAAGGCC AGTCAGAGTG TGAGTAATGA
      G  D  R    V  T  I    T  C  K  A    S  Q  S    V  S  N

501 TGTAGCTTGG TACCAACAGA AGCCAGGGCA GTCTCCTACA CTGCTCATAT
      D  V  A  W    Y  Q  Q    K  P  G    Q  S  P  T    L  L  I

551 CCTATACATC CAGTCGCTAC GCTGGAGTCC CTGATCGCTT CATTGGCAGT
      S  Y  T    S  S  R  Y    A  G  V    P  D  R    F  I  G  S

601 GGATATGGGA CGGATTTCAC TTTCACCATC AGCACTTTGC AGGCTGAAGA
      G  Y  G    T  D  F    T  F  T  I    S  T  L    Q  A  E

651 CCTGGCAGTT TATTTCTGTC AGCAAGATTA TAATTCTCCT CCGACGTTCG
      D  L  A  V    Y  F  C    Q  Q  D    Y  N  S  P    P  T  F

701 GTGGAGGCAC CAAGCTGGAA ATCAAACGG
      G  G  G    T  K  L  E    I  K  R
```

Figure 2

```
1    AAGCTTCCAC CATGGGATGG AGCTGTATCA TCCTCTTCTT GGTAGCAACA
       A  S    T  M  G  W    S  C  I    I  L  F    L  V  A  T

51   GCTACAGGTG TCCACTCCGA GGTCCAGCTT CAGCAGTCTG GACCTGACCT
       A  T  G    V  H  S    E  V  Q  L    Q  Q  S    G  P  D

101  GGTGAAGCCT GGGGCTTCAG TGAAGATATC CTGCAAGGCT TCTGGTTACT
       L  V  K  P    G  A  S    V  K  I    S  C  K  A    S  G  Y

151  CATTCACTGG CTACTACATG CACTGGGTGA AGCAGAGCCA TGGAAAGAGC
       S  F  T    G  Y  Y  M    H  W  V    K  Q  S    H  G  K  S

201  CTTGAGTGGA TTGGACGTAT TAATCCTAAC AATGGTGTTA CTCTCTACAA
       L  E  W    I  G  R    I  N  P  N    N  G  V    T  L  Y

251  CCAGAAATTC AAGGACAAGG CCATATTAAC TGTAGACAAG TCATCCACCA
       N  Q  K  F    K  D  K    A  I  L    T  V  D  K    S  S  T

301  CAGCCTACAT GGAGCTCCGC AGCCTGACAT CTGAGGACTC TGCGGTCTAT
       T  A  Y    M  E  L  R    S  L  T    S  E  D    S  A  V  Y

351  TACTGTGCAA GATCTACTAT GATTACGAAC TATGTTATGG ACTACTGGGG
       Y  C  A    R  S  T    M  I  T  N    Y  V  M    D  Y  W

401  TCAAGTAACC TCAGTCACCG TCTCCTCAGG TGGTGGTGGG AGCGGTGGTG
       G  Q  V  T    S  V  T    V  S  S    G  G  G  G    S  G  G

451  GCGGCACTGG CGGCGGCGGA TCTAGTATTG TGATGACCCA GACTCCCACA
       G  G  T    G  G  G  G    S  S  I    V  M  T    Q  T  P  T

501  TTCCTGCTTG TTTCAGCAGG AGACAGGGTT ACCATAACCT GCAAGGCCAG
       F  L  L    V  S  A    G  D  R  V    T  I  T    C  K  A

551  TCAGAGTGTG AGTAATGATG TAGCTTGGTA CCAACAGAAG CCAGGGCAGT
       S  Q  S  V    S  N  D    V  A  W    Y  Q  Q  K    P  G  Q

601  CTCCTACACT GCTCATATCC TATACATCCA GTCGCTACGC TGGAGTCCCT
       S  P  T    L  L  I  S    Y  T  S    S  R  Y    A  G  V  P

651  GATCGCTTCA TTGGCAGTGG ATATGGGACG GATTTCACTT TCACCATCAG
       D  R  F    I  G  S    G  Y  G  T    D  F  T    F  T  I

701  CACTTTGCAG GCTGAAGACC TGGCAGTTTA TTTCTGTCAG CAAGATTATA
       S  T  L  Q    A  E  D    L  A  V    Y  F  C  Q    Q  D  Y

751  ATTCTCCTCC GACGTTCGGT GGAGGCACCA AGCTGGAAAT CAAACGGGCC
       N  S  P    P  T  F  G    G  G  T    K  L  E    I  K  R  A

801  TCCACCAAGG GCCCATCGGT CTTCCCCCTG GCACCCTCCT CCAAGAGCAC
       S  T  K    G  P  S    V  F  P  L    A  P  S    S  K  S

851  CTCTGGGGGC ACAGCGGCCC TGGGCTGCCT GGTCAAGGAC TACTTCCCCG
       T  S  G  G    T  A  A    L  G  C    L  V  K  D    Y  F  P

901  AACCGGTGAC GGTGTCGTGG AACTCAGGCG CCCTGACCAG CGGCGTGCAC
       E  P  V    T  V  S  W    N  S  G    A  L  T    S  G  V  H
```

```
1     ACCTTCCCGG CTGTCCTACA GTCCTCAGGA CTCTACTCCC TCAGCAGCGT
        T  F  P   A  V  L   Q  S  S  G   L  Y  S   L  S  S

1001  GGTGACCGTG CCCTCCAGCA GCTTGGGCAC CCAGACCTAC ATCTGCAACG
      V  V  T  V   P  S  S   S  L  G   T  Q  T  Y   I  C  N

1051  TGAATCACAA GCCCAGCAAC ACCAAGGTGG ACAAGAAAGT TGAGCCCAAA
      V  N  H   K  P  S  N   T  K  V   D  K  K   V  E  P  K

1101  TCTTGTGACA AAACTCACAC ATGCCCACCG TGCCCAGCAC CTGAACTCCT
        S  C  D   K  T  H   T  C  P  P   C  P  A   P  E  L

1151  GGGGGGACCG TCAGTCTTCC TCTTCCCCCC AAAACCCAAG GACACCCTCA
      L  G  G  P   S  V  F   L  F  P   P  K  P  K   D  T  L

1201  TGATCTCCCG GACCCCTGAG GTCACATGCG TGGTGGTGGA CGTGAGCCAC
        M  I  S   R  T  P  E   V  T  C   V  V  V   D  V  S  H

1251  GAAGACCCTG AGGTCAAGTT CAACTGGTAC GTGGACGGCG TGGAGGTGCA
        E  D  P   E  V  K   F  N  W  Y   V  D  G   V  E  V

1301  TAATGCCAAG ACAAAGCCGC GGGAGGAGCA GTACAACAGC ACGTACCGTG
      H  N  A  K   T  K  P   R  E  E   Q  Y  N  S   T  Y  R

1351  TGGTCAGCGT CCTCACCGTC CTGCACCAGG ACTGGCTGAA TGGCAAGGAG
      V  V  S   V  L  T  V   L  H  Q   D  W  L   N  G  K  E

1401  TACAAGTGCA AGGTCTCCAA CAAAGCCCTC CCAGCCCCCA TCGAGAAAAC
        Y  K  C   K  V  S   N  K  A  L   P  A  P   I  E  K

1451  CATCTCCAAA GCCAAAGGGC AGCCCCGAGA ACCACAGGTG TACACCCTGC
      T  I  S  K   A  K  G   Q  P  R   E  P  Q  V   Y  T  L

1501  CCCCATCCCG GGATGAGCTG ACCAAGAACC AGGTCAGCCT GACCTGCCTG
        P  P  S   R  D  E  L   T  K  N   Q  V  S   L  T  C  L

1551  GTCAAAGGCT TCTATCCCAG CGACATCGCC GTGGAGTGGG AGAGCAATGG
        V  K  G   F  Y  P   S  D  I  A   V  E  W   E  S  N

1601  GCAGCCGGAG AACAACTACA AGACCACGCC TCCCGTGCTG GACTCCGACG
      G  Q  P  E   N  N  Y   K  T  T   P  P  V  L   D  S  D

1651  GCTCCTTCTT CCTCTACAGC AAGCTCACCG TGGACAAGAG CAGGTGGCAG
        G  S  F   F  L  Y  S   K  L  T   V  D  K   S  R  W  Q

1701  CAGGGGAACG TCTTCTCATG CTCCGTGATG CATGAGGCTC TGCACAACCA
        Q  G  N   V  F  S   C  S  V  M   H  E  A   L  H  N

1751  CTACACGCAG AAGAGCCTCT CCCTGTCTCC GGGTAAATGA GTGCGACGGC
      H  Y  T  Q   K  S  L   S  L  S   P  G  K  -   V  R  R

1801  CAAGCTT
        P  S
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